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Discussion

Dr Paul W. M. Fedak *(Calgary, Alberta, Canada)*. There is clear clinical evidence that even with early and successful reperfusion after coronary occlusion, patients will undergo progressive cardiac remodeling that can lead to heart failure. The key molecular and cellular changes that occur after reperfusion are not well known. It gives us a window of opportunity where we could potentially treat patients early during or after reperfusion to prevent maladaptive signals from happening, but we need to understand them better. Your work is important because it leads us in

that direction to better understand mechanistically what is happening with those molecular and cellular changes.

What is unique and important is that you have been able to separate out region- and time-specific changes in a preclinical relevant animal model. What is really important is that you are able to separate ECM interstitial signaling from the cellular changes. It is difficult to present that complexity of data in such a short period of time, and you did a great job. I have a couple of questions for you.

You have designed your experiment to look at mechanistic data and tried to tease out time- and region-specific changes and correlate those events. I note with your data you are trying to correlate MT1-MMP activity and LTB processing, but in the remote area during reperfusion you observed the highest levels of the MMP actively, but you didn't observe the similar changes in the LTB processing. Does that refute your hypothesis?

Dr Eckhouse. I believe the differences seen in MT1-MMP and LTBP-1 activity with microdialysis actually support our hypothesis.

First, in examining MT1-MMP activity with microdialysis during the I/R protocol, there are several possible reasons that could lead to this increased MT1-MMP activity in the remote region. Previous studies from other laboratories have determined that a phosphorylation event occurring at the C-terminus of MT1-MMP can lead to a substrate specificity and directed localization within a cell membrane. From these findings, our research group hypothesizes that post-translational modifications of MT1-MMP in vivo are promoting increased cleavage of the MT1-MMP fluorogenic peptide in a region-dependent manner.

An important question to consider is how are the MT1-MMP and LTBP-1 substrates different? The peptide sequences of each substrate are different. The MT1-MMP fluorogenic peptide is a synthetic amino acid sequence that is hydrolyzed specifically by MT1-MMP in vitro and in vivo. Previous validation studies confirmed that the amino acid sequence is specific for MT1-MMP over other MMPs. The LTBP-1 fluorogenic peptide represents an endogenous substrate sequence that is hydrolyzed by MT1-MMP. Previous in vivo studies have determined that with overexpression of MT1-MMP, there is a decrease in full-length LTBP-1 and an increase in the low-molecular-weight split products of LTBP-1 after MT1-MMP–mediated hydrolysis. Therefore, MT1-MMP differentially processes the 2 synthetic peptides.

The cleavage of the LTBP-1 fluorogenic peptide was specific to the I/R region, which is supported by the increase in the activation of the TGF-ß profibrotic pathway as measured by pSmad2 in the I/R region when compared with the remote region. Although not in the presentation, gene expression of collagen 1A1 by quantitative PCR was increased, which further suggests increased acute activation of the TGF-ß profibrotic pathway within the I/R region that was not measured within the remote region, as demonstrated by no change in collagen 1A1 gene expression and a small increase in pSmad2 protein abundance when compared with the referent control.

Dr Fedak. Can you speculate what the triggers could be here? Could wall stress be the key trigger, and if so, can you think of anything we can do from a surgical point of view early after reperfusion?

Dr Eckhouse. Wall stress has been demonstrated to be an important mechanism in activating MT1-MMP. We have seen this in a recent murine model of pressure overload hypertrophy from our laboratory published by Dr Zile and in in vitro aortic wall stress studies.

The alteration in stress–strain patterns due to an acute I/R event is causing an increase in MT1-MMP activation. Therefore, decreasing wall stress due to I/R remains important.

For a future surgical technique, it will be important to improve stress–strain patterns. Although this may be more relevant to a chronic infarction, improving the thickness of the affected myocardium within the I/R injury region by injections of polymers or other method is currently under investigation. However, there is a lot of area for creativity.

Dr Fedak. Thank you.

Dr Joseph D. Schmoker *(Burlington, Vt)*. To follow up on Paul's question about regional issues, there is a mechanism for uncoupling of TGF-ß via mechanical transduction through the integrin network, I believe. I was wondering if you came across that mechanism and if that could serve as an explanation for why you are seeing this only in the region of I/R?

Dr Eckhouse. I haven't come across that mechanism specifically within this study, but one of my predecessors in the laboratory, Dr Ruddy, looked at the TGF-ß pathway within in vitro studies of aortic wall stress. We are currently investigating increased TGF-ß activation secondary to increased aortic wall stress in models of aortic aneurysms. However, I haven't looked at that within the I/R myocardial model, but that is definitely an area to look at.

Dr Schmoker. My other question relates to miR-133a, which has been used as a biomarker in clinical trials. Could it be that the interstitial levels you are measuring are simply secondary to cell injury or death secondary to reperfusion? Have you looked at that? In other words, do you have histology on your I/R region?

Dr Eckhouse. I don't have histology data to present. We did cell culture studies using primary porcine fibroblasts undergoing simulated in vitro I/R, and what we found when we measured miR-133a within the conditioned media is consistent with our in vivo evidence. When it comes to cell death, previous studies using that same I/R model did not show increased cardiomyocyte death, and we didn't have increased fibroblast cell death with our simulated hypoxia reoxygenation model.

Concurrently, with potential cell death within the I/R region, I would have expected miR-133a extracellularly to increase more with apoptosis and cell death and spillage of the intracellular miR-133a into the interstitial space. However, with I/R within that I/R region, we actually see a decrease during the ischemic time of miR-133a.

Dr Schmoker. Right, but I think it is in the reperfusion episode where you are really going to get the cell injury or death.

One last point. Cardiac myocytes are enriched with miR-133a, but I don't know the relative abundance of that miR-133a in fibroblasts. So I am wondering why you didn't use cardiomyocytes in your cell culture.

Dr Eckhouse. That is a study we are currently doing. We haven't completed that study. We have been able to show a correlation between MT1-MMP levels and miR-133a in myocardial fibroblasts. So we are able to demonstrate that miR-133a is within other cells besides the cardiomyocytes, but we are specifically looking at the cardiomyocytes now.

Dr Stephen McKellar *(Rochester, Minn)*. Elegant study and model. Good presentation. You presented your I/R model here, but have you looked at this or similar phenomena in an infarction model with sampling in the infarct, border zones, and then distantly? This is an elegant way of studying that region. Is that something you could at least speculate on what you might find in that model?

Dr Eckhouse. A previous study evaluated MT1-MMP activity within an infarction model with a subsequent I/R event 2 weeks after the infarction. MT1-MMP activity increased with infarction and then subsequently increased even more after a subsequent I/R event 2 weeks later. However, in regard to miR-133a, we haven't looked at the long-term effects of I/R on miR-133a levels, but it is definitely something we are interested in.

Dr Thomas K. Waddell *(Toronto, Ontario, Canada)*. My question is along the lines of Dr Schmoker's question about the release of miR-133. You mentioned that the exportation was identified as a mechanism of regulation, but on the 2 slides you showed, it was clear that the Y axis was different. So when you measured by PCR the levels of the miR-133 in the interstitium versus the total, what was the scale of that difference? I would put it to you that if the total is a million, then what you are measuring in the interstitium is 10. It probably doesn't reflect regulation. What I would like you to think about is perhaps it is a mechanism of communication—the exportation of miR-133 allows that to be diffused outward. If that is the case, you might actually see increasing penumbra of decreasing miR-133 levels. So you may want to look at your remote area a little bit as proximal and distant remote.

Dr Eckhouse. We didn't look at proximal and distant remote regions of the myocardium. That is an interesting point and definitely a suggestion we will take to the laboratory. Our data with our miR exportation seem to fit nicely with our acute TGF-ß activation. In the remote region, we saw a decrease in the interstitial miR-133a levels, and it remained decreased with reperfusion, which in comparison with total myocardial pools leads us to believe that miR-133a remains elevated intracellularly. Thus, miR-133a can inhibit or attenuate the TGF-ß profibrotic pathway within the remote region. Within the I/R region, the extracellular increase in miR-133a may be associated with a intracellular decrease of miR-133a, which may then allow activation of the TGF-ß profibrotic signaling pathways and promote fibrosis over time.